

Greenhouse bumblebees (*Bombus terrestris*) spread their genes into the wild

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Abstract Bumblebees (*Bombus* spp.) are commonly used for greenhouse pollination of tomatoes and other crop plants. The colonies used for this purpose are provided by commercial bumblebee breeders, which by now operate at a professional company level. As a result of this practice commercially bred bumblebee colonies are transported and used over large distances and national borders, introducing subspecies into non-endemic regions. The question whether and to what extends gene flow between such managed greenhouse and wild bumblebee populations exists, so far has not been addressed. Here we used samples from three greenhouses in Poland and the surrounding populations to address this question. Using microsatellite DNA data we found strong genetic introgression from the sampled greenhouse populations into the adjacent populations. Depending on the analysed population, the number of individuals assigned to the greenhouse populations ranged from 0.08 to 0.47. We also found that more distant populations were much less affected by genetic introgression from the greenhouses.

Keywords *Bombus terrestris* · Genetic introgression · Invasive species · Social insects

Introduction

Bumblebees (*Bombus* spp.) are increasingly used in greenhouses for pollination services, because they are very efficient pollinators that can be handled with great ease. Bumblebees exceed other pollinator species in pollination efficiency, due to their behavioural particularity of “buzz pollination” (Buchmann 1985) especially for many *Solanaceae*, including tomatoes (*Solanum lycopersicum*) or bell peppers (*Capsicum annuum*). An additional advantage for their commercial use in greenhouses is that colonies can be easily bred, handled and transported throughout the year. Colonies can be delivered within a few days on demand, which makes them a perfect insect for indoor pollination. Bumblebee colonies became commercially available for greenhouse pollination from 1980s, and the species of choice in Europe was the buff-tailed bumblebee *B. terrestris*, one of the most common and widespread European species. Even though *B. terrestris* is not the only species used in greenhouse pollination (e.g. *B. impatiens* is the used species in North and Central America) it is definitely the dominant one on the market and is exported to Asia, Australia and South America. The total number of bumblebee colonies reared for greenhouses in 2006 was estimated to be nearly as high as one million (Velthuis and van Doorn 2006).

The downside of the massive exports of bumblebee colonies from Europe into non-endemic areas is that *B. terrestris* has established stable feral populations in New Zealand, Japan, Australia, Tasmania, Chile and Israel (Buttermore 1997; Goulson 2003; Goulson and Hanley 2004; Nagamitsu and Yamagishi 2009; Williams et al. 2009; Williams and Osborne 2009). This quite impressively shows the colonizing capacity of *B. terrestris* which may be facilitated by male haploidy which exposes all

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recessive alleles to selection (Schmid-Hempel et al. 2007). Whatever the reason for this evolutionary success story of *B. terrestris*, the introduction of this bumblebee species to non-endemic regions has raised severe concerns from a conservation perspective. Inoue et al. (2008) report that *B. terrestris* may displace native bumblebee species and the worldwide shipping of colonies may also cause the worldwide spread of their diseases (Goka et al. 2001, 2006) spilling over from greenhouses into wild populations (Colla et al. 2006; Otterstatter and Thomson 2008).

Given these negative effects of *B. terrestris* exportations to non-endemic regions, what is to be expected for Europe, where it is an endemic species? Clearly, from a conservation point of view it would be optimal if bumblebee stocks used in greenhouse pollination were descendants of local populations (Velthuis and van Doorn 2006) to avoid potential negative effects on endemic pollinator communities. Unfortunately this is generally not the case in commercial bumblebee rearing. In fact, the European *B. terrestris* market is dominated by the use of two southern subspecies namely *B. t. dalmatinus* or *B. t. sassaricus*, which have proved to be the most suitable for colony rearing. In spite of evidence that bumblebee workers can fly out of greenhouses (Whittington et al. 2004) many European authorities still assume that it is unlikely that males or young queens escape from greenhouses. And even if bumblebees might escape, they also

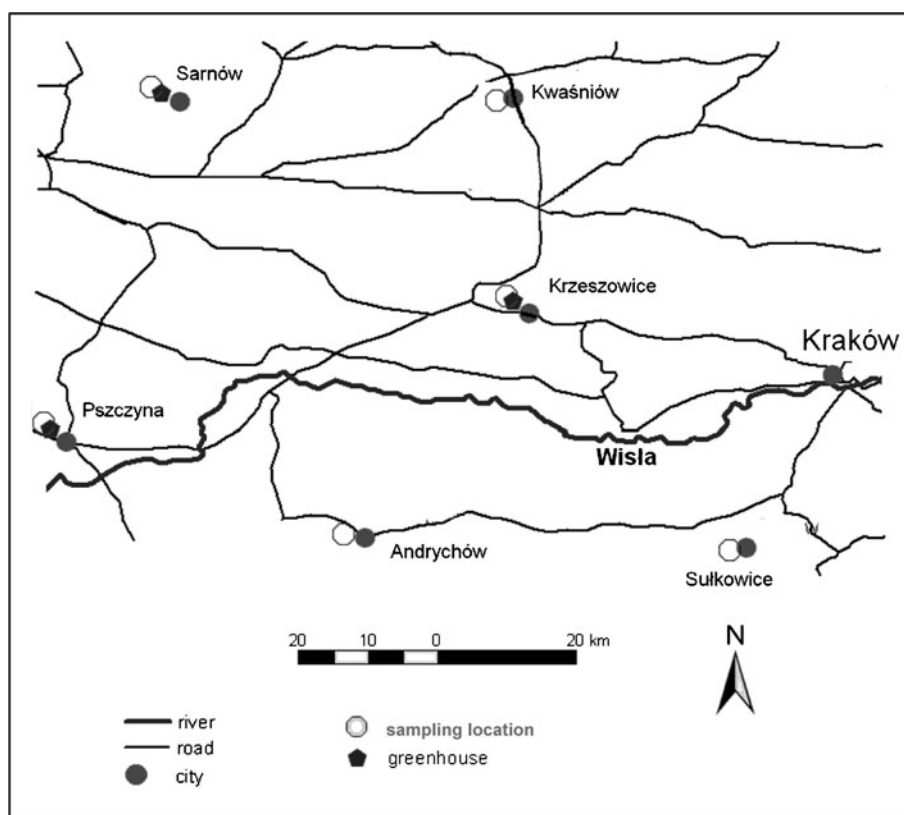
assume that the lack of synchronization with local populations would prevent any interbreeding. Only a few countries (Norway, Turkey, Israel and Spain for Canary Islands) in Europe have imposed import restrictions on non-endemic bumblebee species (Velthuis and van Doorn 2006). In the light of experiences with the invasion of imported bumblebees in Japan (Inoue et al. 2008), there seems to be reason for a more cautious policy of bumblebee usage in greenhouse pollination. Today it is unclear to what extent greenhouse populations interfere with and genetically alters the surrounding wild bumblebee populations. Here we use a population genetic approach to study three selected greenhouse populations of *B. terrestris* and their surrounding wild populations in Southern Poland, to test for a potential introgression of commercial greenhouse bumblebee populations into the wild.

Materials and methods

Sampling

We sampled a total of 588 *B. terrestris* workers from nine populations in Southern Poland (Map 1). Three of these populations were directly collected in greenhouses and thus being of imported, non-endemic origin. Three endemic populations were sampled in the area surrounding the

Map 1 Sampling area and locations near Krakow in Southern Poland



greenhouses and three more endemic populations without any close contact to greenhouses at a distance of at least 30 km away. The sample sizes, names, GPS data and abbreviations of the populations are given in Table 1. All sampled workers were stored in 95% ethanol at -20°C until DNA extraction.

Genotyping

Total DNA was extracted from one leg of each of the sampled workers following a modified Chelex extraction protocol as described by Walsh et al. (1991). All individuals were genotyped at four microsatellite loci (B11, B96, B124, B126; Estoup et al. 1993, 1995) following standard polymerase chain reaction (PCR) protocols in automated DNA capillary sequencers (MegaBACE 1000) according to manufacturer's instructions.

Population genetic analysis

Before embarking on classical population genetic analyses, it is important to assign each individual worker to its putative mother colony resulting in a mother and father genotype for each colony. This way biases due to sampling multiple workers of the same colony can be avoided. Queen and father drone genotypes were inferred using the COLONY 1.3 algorithm (Wang 2004) performing four

runs with different seed numbers and using those genotype assignments with the highest log probability for further analyses. Based on the mother/father genotypes we calculated the observed and expected heterozygosities, deviation from Hardy–Weinberg equilibrium, pairwise F_{ST} values and population differentiation, using the Excel Microsatellite Toolkit 3.1.1 (Park 2001) and Genepop (Version: *Genepop on the Web*; Raymond and Rousset 1995). The number of alleles and the allelic richness was calculated using HP-Rare 2.0 (Kalinowski 2004, 2005).

To infer the degree of introgression, we determined the number of individuals sampled in the surrounding populations, but which were assigned to the greenhouse populations. The individual workers were assigned using BAPS 2.0 (Corander et al. 2004). We used the trained clustering option of the software (Corander et al. 2008) with the greenhouse populations as fixed clusters of known origin. Since two of the populations (K-GH and P-GH) were originating from the same supplier they were pooled in one cluster, while the remaining greenhouse population S-GH formed the second fixed cluster. All other populations were treated as of unknown origin, thus it was possible to test which of the endemic populations' colonies were clustered to one of the greenhouse clusters. To infer the degree of introgression we used ten replicate runs on BAPS, using the mean and standard deviation of the number of individuals assigned to the greenhouses as our final estimate of introgression.

Table 1 Given are the names, abbreviations and GPS positions of the sampling locations, followed by the sampling size (n), number of inferred colonies (n_c), observed (H_o) and expected (H_e) heterozygosities, average number of alleles (A) and average allelic richness (A_R)

Name	Abrv.	GPS	n	n_c	H_o	H_e	A	A_R	Intr. K/P-GH	Intr. S-GH	Intr. total
Krzeszowice GH	K-GH	50.135°N 19.647°E	187	23	0.57 ± 0.05	0.73 ± 0.05	8.5 ± 2.4	6.7 ± 1.6	n.a.	n.a.	n.a.
Pszczyna GH	P-GH	49.964°N 18.929°E	133	20	0.67 ± 0.05	0.75 ± 0.06	8.5 ± 2.1	7.2 ± 2.0	n.a.	n.a.	n.a.
Sarna GH	S-GH	50.381°N 19.158°E	30	13	0.82 ± 0.05	0.75 ± 0.06	8.5 ± 2.6	7.6 ± 2.1	n.a.	n.a.	n.a.
Krzeszowice	K	50.142°N 19.658°E	59	31	0.77 ± 0.04	0.75 ± 0.06	12.3 ± 2.6	8.3 ± 2.0	0.23 ± 0.04	0.10 ± 0.01	0.33 ± 0.03
Pszczyna	P	49.957°N 18.914°E	39	16	0.75 ± 0.06	0.70 ± 0.14	11.3 ± 5.1	8.8 ± 3.5	0.27 ± 0.05	0.06 ± 0.04	0.32 ± 0.04
Sarna	S	50.390°N 19.161°E	45	16	0.86 ± 0.05	0.73 ± 0.04	8.3 ± 2.1	7.2 ± 1.7	0.20 ± 0.06	0.28 ± 0.11	0.47 ± 0.08
Andrychow	A-C	49.847°N 19.305°E	21	11	0.75 ± 0.07	0.66 ± 0.10	8.3 ± 4.2	7.5 ± 3.3	0.07 ± 0.05	0.14 ± 0.06	0.21 ± 0.05
Sulkowice	S-C	49.882°N 19.805°E	21	9	0.87 ± 0.06	0.76 ± 0.03	8.5 ± 1.9	8.3 ± 1.7	0.00 ± 0.00	0.08 ± 0.05	0.08 ± 0.02
Kwasniow	K-C	50.370°N 19.604°E	53	22	0.83 ± 0.04	0.72 ± 0.06	9.8 ± 4.6	7.5 ± 2.9	0.06 ± 0.04	0.02 ± 0.02	0.08 ± 0.03

The last three columns give the average degree of introgression (Intr.) from the greenhouse population, either K-GH and P-GH or S-GH, into a given population (n.a. = not applicable)

Results

Based on the sample of 588 workers from the nine populations we inferred a total of 161 colonies, each represented by one queen and male genotype. The number of colonies inferred for each population, the number of alleles and the allelic richness in the parental population (based on male and queen genotypes) are given in Table 1. The observed heterozygosities ranged from 0.57 in the K-GH population up to 0.87 in the S-C population, all observed and expected heterozygosity values are given in Table 1. Only one population (K) deviated significantly from Hardy–Weinberg proportions, which however did not remain significant after Bonferroni correction for multiple tests. The allelic richness over all loci ranged from 6.7 ± 1.6 (mean \pm SD) in the K-GH population up to 8.8 ± 3.5 in the P population, all values of the observed number of alleles and the allelic richness are given in Table 1. The pairwise F_{ST} values ranged from 0.02 to 0.22, with the lowest value for the two greenhouse populations provided by the same commercial bumblebee breeder. The test for population differentiation revealed that only 4 out of the 36 pairwise tests showed no significant population differentiation after Bonferroni correction for multiple tests. These were the Populations Kr-Gh/P-Gh, S-Gh/S, K/P and P/S. The F_{ST} values and the significance of the Fisher's exact test for population differentiation are given in Table 2.

The estimated degree of individuals assigned to the greenhouse populations ranged from 0.08 in the K-C and S-C populations up to 0.47 in the S population. Moreover the more distant control populations showed significantly lower average amount of individuals assigned to the greenhouses (0.13 ± 0.08) than the close by populations (0.38 ± 0.08). The detailed percentages of individuals assigned to the greenhouse populations are given in Table 1.

Table 2 Given are the pairwise F_{ST} values (lower diagonal) and the significance of the pairwise test for population differentiation (upper diagonal, * = significant differentiation)

	K-GH	P-GH	S-GH	K	P	S	A-C	S-C	K-C
K-GH	–	n.s.	*	*	*	*	*	*	*
P-GH	0.02	–	*	*	*	*	*	*	*
S-GH	0.06	0.07	–	*	*	n.s.	*	*	*
K	0.05	0.06	0.05	–	n.s.	*	*	*	*
P	0.05	0.09	0.09	0.03	–	n.s.	*	*	*
S	0.07	0.10	0.04	0.07	0.07	–	*	*	*
A-C	0.12	0.14	0.12	0.07	0.10	0.16	–	*	*
S-C	0.12	0.13	0.11	0.09	0.16	0.12	0.16	–	*
K-C	0.18	0.18	0.18	0.18	0.22	0.19	0.18	0.18	–

Discussion

In this study we estimated the degree of genetic introgression from *Bombus terrestris* greenhouse populations into feral populations by assigning individuals to their potential population of origin. The percentage of individuals assigned to the greenhouse populations was very high in the nearby wild populations surrounding the greenhouses. The percentage of introgression ranged from 33 to 47% and thus was significantly higher than the percentage estimated for the more distant control populations, which however still had between 8 and 21% of individuals assigned to the greenhouse populations. When taking a closer look at the introgression pattern in the nearby wild populations, the degree of introgression corresponds to the greenhouse population they are neighbouring. The populations K and P have a much higher degree of introgression from the K-GH/P-GH population complex than from the S-GH population, while the opposite is true for the S population which neighbours to the S-GH population (Table 1).

The test for population differentiation revealed the K-GH and P-GH populations did not differ significantly from each other, which is expected, since the bumblebees used in these two greenhouses are from the same supplier, and are thus from a similar or the same breeding stock. Also within the group of the nearby populations, K, P and S, three pairwise comparisons were not significant, even though there is no obvious consistent pattern of genetic differentiation. The high similarity among the populations may result from repeated introgressions from the greenhouse populations in the past to such a degree that three of the pairwise tests for genetic differentiation fail to detect any sub-structuring. Another reason for the three non significant pairwise tests might also be a lack of power due to our usage of four microsatellite markers. A higher number of markers might have resulted in a better resolution and would thus be desirable for future studies.

The pairwise F_{ST} values provide a similar picture where the three greenhouse populations show the lowest degree of genetic differentiation (average $F_{ST} = 0.05$), with the lowest of all detected values ($F_{ST} = 0.02$) between the two populations from the same supplier, the populations K-GH and P-GH. Substructuring is however also low among the K, P and S populations with an average $F_{ST} = 0.06$. The highest pairwise F_{ST} values were found between the control populations, A-C, S-C and K-C (average $F_{ST} = 0.15$), with K-C as the genetically most differentiated population to all other ones (average $F_{ST} = 0.19$).

Interestingly the two populations with lowest allelic richness and the lowest value for the observed heterozygosity are the two populations K-GH and P-GH populations. While this might be a sign of inbreeding due to the breeding of bumblebees from a limited number of queens,

there is no deviation from Hardy–Weinberg and our study was generally not designed to detect such effects. So, it remains difficult to judge whether the commercial breeding had already an inbreeding effect on the greenhouse populations.

We used an assignment test based on trained clustering to infer the percentage of individuals in the populations for the estimation of genetic introgression. The genetic characteristics suggest that introgressed individuals are direct descendents from the greenhouse populations, either offspring of gynes which had escaped from the greenhouses or workers which were conducting foraging flights outside the greenhouses. Although we can certainly not exclude the latter we expect this to be a rare phenomenon since there is ample forage in the greenhouses. Worker bees typically forage at nearby food sources and not necessarily leave the greenhouse. In the more distant control populations this phenomenon can be excluded because they are outside the foraging range of the greenhouse colonies which does normally not exceed 0.8 km (Wolf and Moritz 2008).

The degree of introgression in the more distant populations was as low as 7% and thus markedly lower than around the greenhouses. However these populations were chosen under the assumption to be not affected by introgression. Therefore we cannot exclude that the estimated introgression is an artefact of the estimation procedure, which would mean that also our estimates for the nearby populations are overrated. However even in this case where we would assume an average false introgression rate of 12.1% (which is the average for the distant populations), our estimates for the nearby populations would still range between 20 and 35%. In contrast, if we consider the genetic introgression results for the distant populations as valid, they imply that genes from the greenhouse populations already have spread much further than initially expected. This definitely would be the worst case scenario from a conservation perspective.

Regardless of scenario or statistical pitfalls, our results strongly suggest that greenhouse bumblebees spread their genes into feral conspecific populations. This is in line with previous studies, which already showed that imported greenhouse bumblebees can become an invasive species in competition with native species (e.g. Goulson and Hanley 2004; Hingston 2006; Inoue et al. 2008; Williams and Osborne 2009). In our case of conspecific introgression, the effects and consequences of genetic spillover from non-endemic subspecies to endemic ones are harder to predict. The genetic introgression will surely alter the genetic structure of a given population, which in turn may lead to the loss of specific traits and local adaptations. Introgression will depend on the scale of import and the ratio of wild to greenhouse colonies. Today we do not know the

tolerable threshold value of influx of non-endemic genes to maintain stable local population.

In conclusion our results imply that bumblebees used in greenhouses can not only trigger a classical biological invasion but are also capable of changing the genetic structure of conspecific endemic subspecies. Since the consequences of this genetic spillover are hard to predict and might have potential negative affects for the conservation efforts of bumblebees, it seems prudent to handle and manage bumblebee imports with caution in regions where *B. terrestris* is endemic.

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